



Published in final edited form as:

*J Cell Biochem.* 2016 April ; 117(4): 853–859. doi:10.1002/jcb.25381.

## Golgi Associated HIF1a Serves as a Reserve in Melanoma Cells

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### Abstract

Hypoxia-inducible factor-1alpha (HIF1a) is a key transcriptional regulator that enables cellular metabolic adaptation to low levels of oxygen. Multiple mechanisms, including lysosomal degradation, control the levels of HIF1a protein. Here we show that HIF1a protein degradation is resistant to lysosomal inhibition and that HIF1a is associated with the Golgi compartment in melanoma cells. Although pharmacological inhibitors of prolyl hydroxylation, neddylation and the proteasome inhibited degradation of HIF1a, attenuation of lysosomal activity with chloroquine did not alter the levels of HIF1a or its association with Golgi. Pharmacological disruption of Golgi resulted in nuclear accumulation of HIF1a. However, blockade of ER-Golgi protein transport in hypoxia reduced the transcript levels of HIF1a target genes. These findings suggest a possible role for the oxygen-dependent protein folding process from the ER-Golgi compartment in fine-tuning HIF1a transcriptional output.

### Keywords

HIF1a; GOLGI; MELANOMA; ENDOPLASMIC RETICULUM; LYSOSOME; HYPOXIA

Low levels of oxygen dictate the metabolic adaptation of cells in metazoans. The transcription factor HIF1a is central to this metabolic flexibility, and its protein stability is tightly coupled to the levels of oxygen. Physiological oxygen levels trigger prolyl hydroxylases that lead to hydroxylation of specific prolines (Pro402 and Pro564) of HIF1a [Schofield and Ratcliffe, 2004; Semenza, 2009]. This modification enables the recruitment of von Hippel-Lindau (VHL) complex followed by ubiquitination and rapid proteosomal degradation of the HIF1a protein [Ivan et al., 2001; Jaakkola et al., 2001]. Conversely, hypoxic conditions limit the prolyl hydroxylase activity, leading to HIF1a protein accumulation and translocation into the nucleus. This nuclear HIF1a activates transcription of genes such as PDK1, LDHA, BNIP3 and BNIP3L that in turn switch the cells from generating ATP from oxidative phosphorylation (OxPhos) to glycolysis [Semenza, 2013].

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Rapid turnover of HIF1 $\alpha$  protein is controlled by multiple mechanisms. Prolyl hydroxylation primes HIF1 $\alpha$  protein for recognition by the VHL complex [Soucy et al., 2009]. VHL is a part of VCB-Cul2 protein degradation scaffold, which requires neddylation for its activation [Stebbins et al., 1999]. Pharmacological inhibition of prolyl hydroxylases or blockade of neddylation stabilizes HIF1 $\alpha$  protein, suggesting that a complex coordinated cascade of post-translational modifications regulates HIF1 $\alpha$  stability.

Autophagy is an evolutionarily conserved process by which proteins are directed to the lysosome for degradation. Based on the mechanism of substrate selection, autophagy is defined as either microautophagy, macroautophagy, or chaperon-mediated autophagy (CMA) [Orvedahl and Levine, 2009]. Typically, macroautophagy targets organelles, microautophagy works via direct capture of substrates into the lysosome, while CMA selectively eliminates soluble proteins. In each case, the substrates are delivered to the lysosome for degradation [Glick et al., 2010]. Macroautophagy is known to promote tumor cell growth in nutrient limited environments, and inhibition of macroautophagy with chloroquine as a possible anti-cancer strategy has been reported [Amaravadi et al., 2011; Yang et al., 2011]. However, metabolic plasticity of tumor cells poses challenges to targeting the lysosome. It was reported that HIF1 $\alpha$  binds to CMA mediators HSC70 and LAMP2A for transport and consequent destabilization in the lysosome [Hubbi et al., 2013]. Furthermore, pharmacological approaches that increase or decrease the activity of lysosomal proteases modulated the degradation of HIF1 $\alpha$  protein in a manner congruent with the lysosomal activity [Hubbi et al., 2013].

We previously reported that chloroquine promotes apoptosis in melanoma cells but not in epithelial tumor cells [Lakhter et al., 2013]. This led us to examine the effects of chloroquine on HIF1 $\alpha$  protein stability in melanoma. Consistent with the previous report [Hubbi et al., 2013], chloroquine inhibits lysosomal destabilization of HIF1 $\alpha$  in epithelial tumor cells; however, this was not the case in melanoma cells. We found that in contrast to epithelial tumor cells, HIF1 $\alpha$  protein is associated with the Golgi compartment in melanoma and disruption of this association reduces its transcriptional activity in hypoxia.

## RESULTS

### CHLOROQUINE INHIBITS HIF1 $\alpha$ DEGRADATION IN EPITHELIAL TUMOR CELLS BUT NOT IN MELANOMA

To test whether chloroquine impacts HIF1 $\alpha$  protein stability in melanoma similar to previously reported effects on non-melanoma tumor cells [Hubbi et al., 2013], MEL526, RPMI8322 and MEL2664 melanoma cells and PC3 prostate cancer cells and HT1080 fibrosarcoma line were exposed to chloroquine or deferoxamine, and the levels of HIF1 $\alpha$  were assessed. Regardless of the cell type, deferoxamine, a commonly used hypoxia mimetic, stabilized the HIF1 $\alpha$  protein. In agreement with a previous report [Hubbi et al., 2013], chloroquine exposure of PC3 and HT1080 cells showed accumulation of HIF1 $\alpha$  and the lysosomal substrate LC3II (Fig. 1A, B). Although chloroquine inhibited lysosomal degradation of LC3II in melanoma cells, it had minimal to no effect on HIF1 $\alpha$  protein levels (Fig. 1C–E). These results prompted us to test whether known modulators of HIF1 $\alpha$  protein stability differ in melanoma. To test this, MEL526 or PC3 cells were exposed to prolyl

hydroxylase inhibitor DMOG, deferoxamine, the neddylation inhibitor MLN4924, or the proteosomal inhibitor MG132, and HIF1 $\alpha$  protein levels were determined. Inhibition of prolyl hydroxylation, neddylation, or ubiquitination stabilized HIF1 $\alpha$  in non-melanoma and melanoma cells alike, however, chloroquine inhibited HIF1 $\alpha$  degradation only in non-melanoma cells (Fig. 1F, G).

### **HIF1 $\alpha$ ASSOCIATES WITH GOLGI IN MELANOMA**

The lack of HIF1 $\alpha$  stabilization by lysosomal inhibition led us to a hypothesis that HIF1 $\alpha$  localization in melanoma may differ from epithelial tumor cells. To test this, MEL526 and PC3 cells were treated with either vehicle or chloroquine, and localization of HIF1 $\alpha$  was determined by immunofluorescence. HIF1 $\alpha$  immunostaining from vehicle treated cells revealed a diffuse cytoplasmic localization in PC3 cells and a discrete pattern of localization in MEL526, reminiscent of the Golgi apparatus (Fig. 2A, B). Co-staining with the antibodies against the Golgi marker GM130 showed co-localization with HIF1 $\alpha$  in melanoma (Fig. 2B). Upon exposure to chloroquine, PC3 cells showed increased HIF1 $\alpha$  immunostaining in the nucleus whereas the Golgi association observed in melanoma cells was insensitive to chloroquine exposure. However, inhibition of prolyl hydroxylases with DMOG led to nuclear accumulation of HIF1 $\alpha$  in both melanoma and non-melanoma cell types.

### **GOLGI-ASSOCIATED HIF1 $\alpha$ SERVES AS A RESERVE**

Brefeldin A (BFA) is an antibiotic that blocks protein transport from the endoplasmic reticulum (ER) to Golgi. To test whether inhibition of ER-Golgi transport alters HIF1 $\alpha$  localization, MEL526 cells were treated with vehicle or BFA and HIF1 $\alpha$  localization was determined. As evident from BFA-mediated loss of discrete GM130 localization suggesting the disruption of Golgi structure, BFA treatment induced HIF1 $\alpha$  protein accumulation in the nucleus in MEL526 cells (Fig. 3A). We hypothesized that Golgi may play an important role in transcriptional activity of HIF1 $\alpha$  in melanoma cells. MEL526 and PC3 cells were treated with vehicle or BFA in 1% oxygen and HIF1 $\alpha$  target gene expression was determined. Although CA9 expression was induced in hypoxia, BFA did not change the transcript levels of HIF1 $\alpha$  targets CA9, LDHA, LDHB and PDK4 even after six hours of hypoxia in PC3 cells (Fig. 3B). However, induction of these transcripts was significantly reduced in the presence of BFA under hypoxic conditions in MEL526 cells. These results suggest that an optimal transcriptional activity of HIF1 $\alpha$  requires ER-Golgi protein transport.

## **DISCUSSION**

The present study demonstrates that unlike in epithelial tumor cells, HIF1 $\alpha$  protein is associated with the Golgi compartment in melanoma. Pharmacological disruption of ER-Golgi transport led to the nuclear localization of HIF1 $\alpha$ . This suggests that melanoma cells harbor a novel mechanism to target a portion of HIF1 $\alpha$  protein to the nucleus via the Golgi compartment. We found that disruption of ER-Golgi transport reduced HIF1 $\alpha$  transcriptional activity in melanoma cells. Collectively, these results suggest that Golgi plays an important role in regulation of HIF1 $\alpha$  activity in melanoma.

Nearly one third of newly synthesized proteins pass through the Golgi apparatus before reaching their destination. Therefore, the Golgi apparatus has been viewed as a protein sorting station [Guo et al., 2014]. In addition to its role in sorting proteins to various destinations, Golgi has been recognized for its role in storage, ensuring proper folding of proteins as well as post-translational modifications [Baumann, 2014]. Therefore, questions arise as to why melanoma cells direct HIF1 $\alpha$  protein to Golgi and whether a specific HIF1 $\alpha$  modification takes place at the Golgi or if it merely acts as immediate source of HIF1 $\alpha$  protein supply in order to rapidly respond to a metabolic change. However, it is clear that melanoma cells exposed to BFA promote HIF1 $\alpha$  localization to the nucleus suggesting that the Golgi-associated HIF1 $\alpha$  can be mobilized to the nucleus similar to the effects of prolyl hydroxylase inhibition. These results invoke the possible existence of several distinct pools of HIF1 $\alpha$  in cells that undergo different modes of degradation. The majority of HIF proteins undergo conventional prolyl hydroxylation and subsequent proteosomal degradation, while a pool of HIF1 $\alpha$  protein in melanoma cells is directed to the ER-Golgi pathway. Disruption of HIF1 $\alpha$  localization to Golgi in DMOG-treated cells suggests that prolyl hydroxylation likely precedes HIF1 $\alpha$  accumulation at the Golgi compartment. Analysis of HIF1 $\alpha$  transcriptional activity under hypoxic conditions reveals that intervention of ER-Golgi protein transport reduces the HIF1 $\alpha$  transcriptional response. Although HIF1 $\alpha$  protein lacks ER retention sequence, a substantial amount of HIF localization to the ER is mediated by its interaction with VHL [Schoenfeld et al., 2001]. A 3D two-photon confocal laser microscopy coupled co-localization study revealed that HIF protein localized to the ER was regulated by the local Fenton reaction at the ER [Liu et al., 2004]. A commonly used Golgi marker GM130 is a peripheral cytoplasmic protein that binds tightly to the Golgi membrane as a part of larger oligomeric complex [Nakamura et al., 1995]. Based on the observed co-localization of HIF with GM130, it is likely that HIF bound to the cytosolic side of Golgi in melanoma cells. Several proteins are known to move from the ER-Golgi compartment to the nucleus. For example, SOK1 translocates from the cytoplasmic side of Golgi to the nucleus and induces cell death in response to chemical anoxia [Nogueira et al., 2008]. Studies from yeast and mammalian cells suggest that proteins such as ATF6, PKA, SREBP2 and PIK1 are mobilized from the cytoplasmic side of Golgi to the nucleus [Constantinescu et al., 1999; Horton et al., 2002; Demmel et al., 2008; Guan et al., 2011]. Consistent with these studies, our observations suggest that melanoma presents a case where Golgi plays a critical role in regulation of HIF1 $\alpha$  transcriptional activity.

An important consideration in hypoxia is the limited availability of oxygen, which is crucial for several biochemical processes in the cell. For instance, protein folding requires disulfide bond formation. In the presence of molecular oxygen, protein disulfide isomerase cooperates with endoplasmic reticulum oxidoreductase-1 (Ero1) to ensure optimal protein folding [Tu et al., 2000]. Although it is not known whether Ero1-La directly acts on HIF1 $\alpha$ , it has been reported that Ero1-La cooperates with HIF1 $\alpha$  in hypoxic adaptation [May et al., 2005]. It was recently reported that disulfide bond formation in the ER requires molecular oxygen [Koritzinsky et al., 2013]. Importantly, there are 11 Cysteine residues in the dimerization domain of HIF1 $\alpha$  that may require oxygen for disulfide bond formation to enable optional folding of the PAS domains. Given that suppression of the ER-Golgi transport resulted in reduced HIF1 $\alpha$  transcriptional output in melanoma cells, it is plausible that differential

folding, determined by the levels of oxygen in the ER, may dictate the dimerization capacity and promoter selection by the HIF1a transcription complex. It is conceivable that the structural variations in folding of the dimerization domain may fine tune transcriptional functions of HIF1a. Failure in protein folding results in unfolded protein response and if it exceeds a certain threshold, cells undergo programmed cell death [Sano and Reed, 2013]. Therefore, it is tempting to speculate that melanoma cells utilize HIF1a as a molecular sensor to monitor not only oxygen levels, but also the ER protein folding capacity, in order to avoid cell death. It is likely that this is a safeguard mechanism that ensures adaptation to hypoxic conditions, while avoiding deleterious effects of hypoxia, by synchronizing protein-folding capacity to low oxygen levels during the metabolic shift to glycolysis.

In light of our previous report demonstrating that a recurrent glioma mutation found in dimerization domain of HIF1a suppresses mitochondrial respiration [Lakhter et al., 2014], and given the potential role of the ER oxygen levels in oxidative folding of dimerization domain, a better understanding of the this domain in the context of ER-Golgi localization may have implications in patho-physiology of cancer, neurodegenerative diseases and diabetes.

## EXPERIMENTAL PROCEDURES

### CELL CULTURE

Cell lines were maintained at 37 °C at 5% CO<sub>2</sub> in RPMI 1640 or DMEM culture media (Sigma) supplemented with 10% fetal bovine serum (Sigma), 50 Uml<sup>-1</sup> penicillin and 50 µgml<sup>-1</sup> streptomycin (Life Technologies). MEL526, PC3 cell lines in RPMI 1640, and HT108, RPMI 8322, MEL2664 cell lines in DMEM maintained. Hypoxic conditions (1% O<sub>2</sub>) were achieved in a Ruskin InvivO<sub>2</sub> 400 hypoxia chamber, by supplementing ambient air with balanced N<sub>2</sub> and CO<sub>2</sub>.

### IMMUNOFLUORESCENCE

Cells were grown on glass chamber slides (Millipore) and treated with indicated compounds. After treatment, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.05% Triton X-100 for 5 min, and blocked with 5% protease-free BSA for 1 h. Samples were incubated with primary antibody in 2% protease-free BSA for 1 h, and incubated with CF dye-conjugated secondary antibody (Biotium) for 1 h. Imaging was captured on Eclipse 80i fluorescent microscope (Nikon) with Retiga Exi (Qimaging) camera.

### SDS-PAGE, WESTERN BLOTTING, AND ANTIBODIES

Whole-cell extracts were prepared in urea buffer (6 M urea, 100 mM sodium dihydrophosphate, 10 mM Tris pH 8). SDS-PAGE was performed using TGX gradient gels (Bio-Rad) and transferred onto PVDF membranes (Millipore) using TransBlot SD semi-dry transfer apparatus (Bio-Rad). The blots were probed with following antibodies: HIF1a (R&D Systems), LC3 (Novus), and tubulin (Sigma). Blot images were captured on ImageQuant LAS 4000 digital imaging system (GE Healthcare).

## REAGENTS

MLN4924 was purchased from BostonBiochem, chloroquine and DMOG from Sigma, all other chemicals were purchased from Cayman Chemical. HIF1a antibodies were purchased from R&D Systems, and GM130 from Cell Signaling Technology, LC3 from Novus, and tubulin from Sigma.

## QUANTITATIVE REAL-TIME PCR

RNA was extracted using NucleoSpin II RNA extraction kit (Clontech) and followed by reverse transcription with RNA to cDNA EcoDry Premix (Clontech). Quantitative PCR was done on CFX 96 Real-Time PCR Detection System instrument (Bio-Rad) using SsoFast EvaGreen Supermix (Bio-Rad), and relative expression was calculated using ddCt method with target transcript normalized to that of RNPII and 18SRNA. Following oligonucleotide primer sequences were used: 18S RNA ACCCGTTGAACCCATTCGTGA (forward) and GCCTCACTAAACCATCCAATCGG (reverse), CA9 TCCCTGCCGAGATCCACGTG (forward) and TTTCTTCCGGGCCCTCCTCC(reverse), LDHATTCTAAGGAAAAGGCTGCCA (forward) and ATGGCCTGTGCCATCAGTAT (reverse), LDHB TCCATGTATCCTCAATGCCCCG (forward) and TCTGCACTTTTCTTGAGCTGAGC (reverse), PDK4 TCTACTCGGATGCTGATGAACCA (forward) and ACCACTGCTACCACATCACAGT (reverse).

## Acknowledgments

Grant sponsor: CTSI Independent Scientist Award; Grant sponsor: CTSI Core award; Grant sponsor: Institutional ACS pilot award.

We thank Zalfa Abdel-Malek for critical reagents. This work was supported in part by the CTSI Independent Scientist Award and CTSI Core award to SRN as well as Institutional ACS pilot award to SRN.

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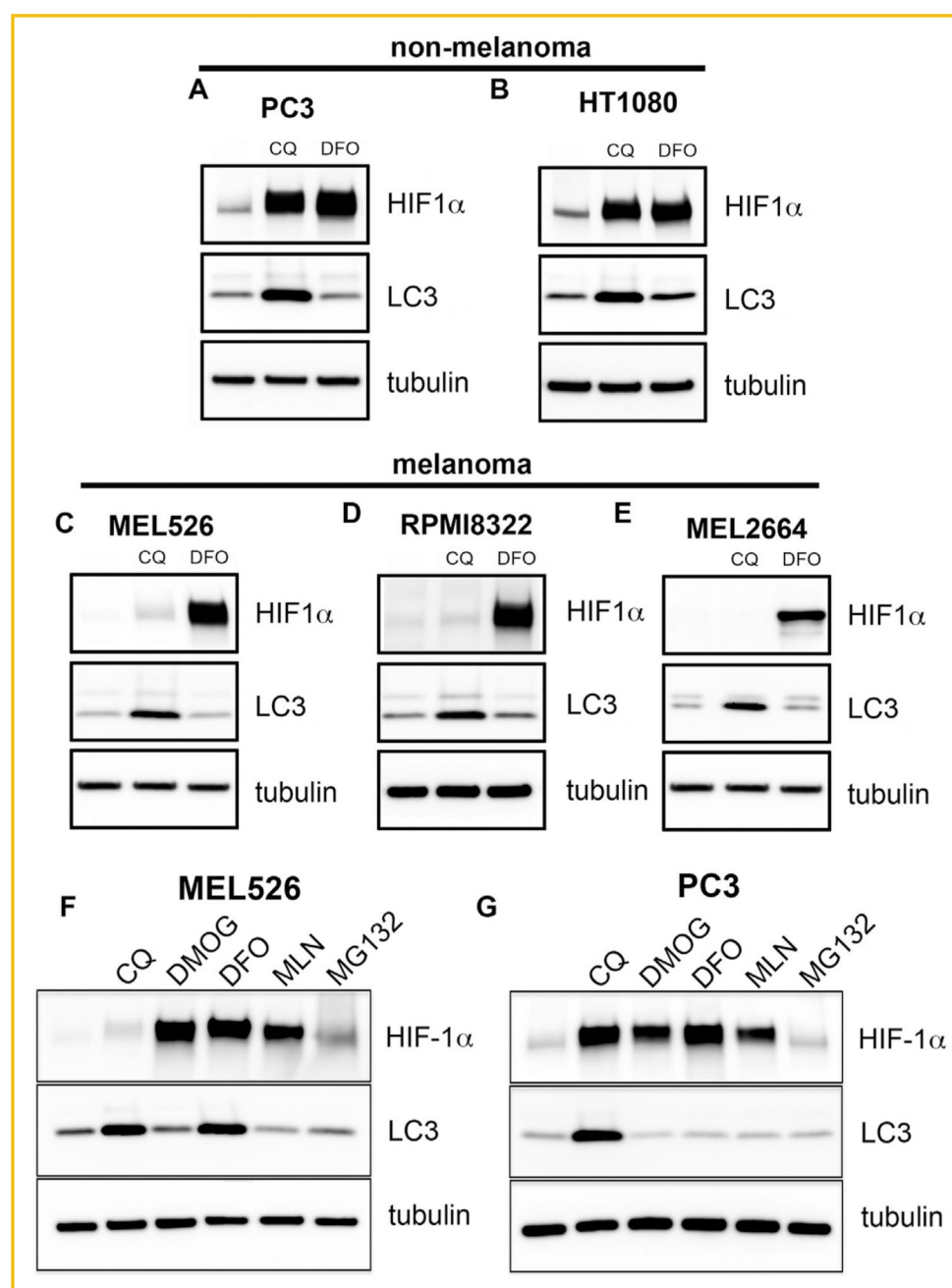
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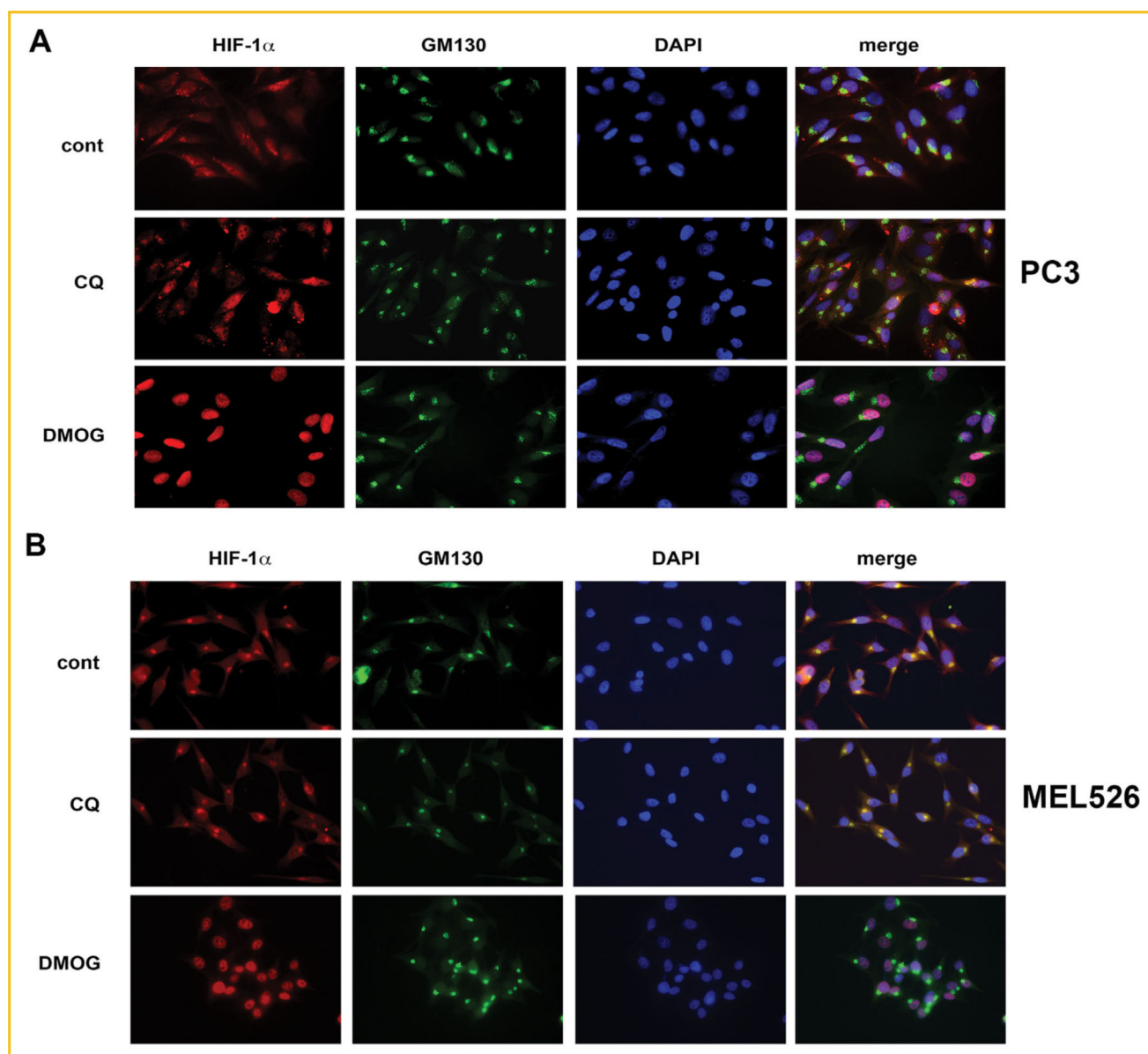
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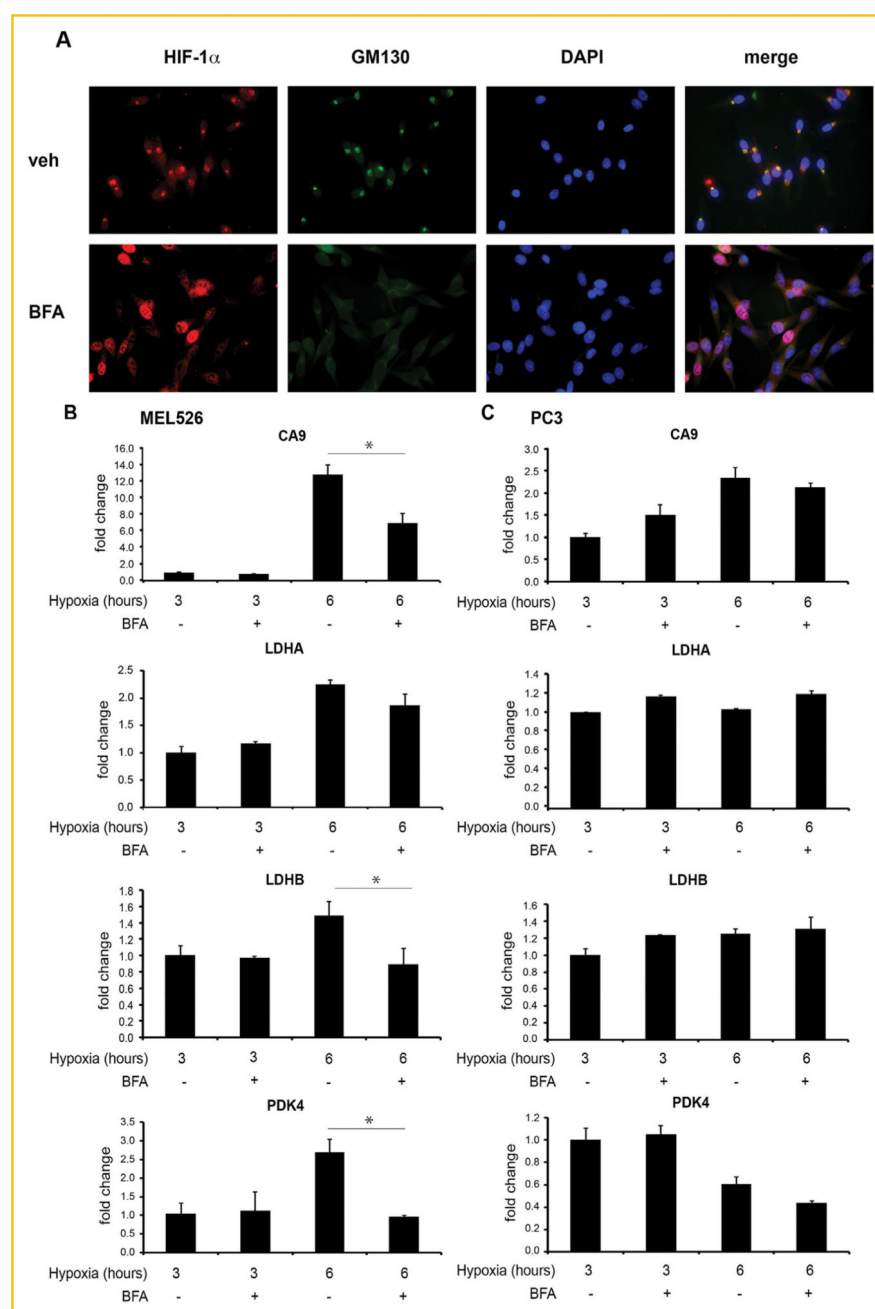


**Fig. 1.**

Chloroquine inhibits degradation of HIF1α in non-melanoma but not in melanoma cells. (A and B) PC3 (prostate cancer) and HT1080 (osteosarcoma) cell lines were treated with 50 μM chloroquine (CQ) or 100 μM deferoxamine (DFO) for 8 h. Cell extracts (20 μg/sample) were resolved on a SDS-PAGE and blotted with HIF1α, LC3 and tubulin antibodies. (C, D and E) MEL526, RPMI8322, MEL2664 melanoma lines cell lines exposed to CQ or DFO and cell lysates were probed with indicated antibodies. (F and G) MEL526 and PC3 cell lines were exposed to CQ (50 μM), DMOG (500 μM), DFO (100 μM), MLN4924 (10 μM) and MG132 (5 μM) for 8 h and cell lysates were blotted with HIF1α, LC3 and tubulin antibodies.

**Fig. 2.**

HIF1α protein associates with Golgi in melanoma cells. (A) PC3 cells and (B) MEL526 cells were exposed to vehicle or 50  $\mu$ M CQ for 8 h and the cells were subjected to immunostaining. Representative fluorescence microscopy images from three independent experiments reveal the subcellular localization of HIF1α (red) and the Golgi marker GM130 (green). The nucleus is stained with DAPI (blue).

**Fig. 3.**

Golgi associated HIF1a serves as a reserve in melanoma. (A) MEL526 cells exposed to vehicle or 2.5  $\mu$ g/ml brefeldin A and the localization of HIF1a (red) and GM130 (green) were visualized by immunofluorescence microscopy and a representative image from three independent experiments shown. (B) MEL526 cells were treated with vehicle or 2.5  $\mu$ g/ml BFA in 1% oxygen and the transcript levels of HIF1a target genes were quantified by quantitative real-time RT-PCR. Data in all graphs are shown as mean  $\pm$  SD. \*  $P < 0.05$  by Student's  $t$  test.